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(54) Title: METHODS FOR ENHANCING OR REDUCING PREIMPLANTATION EMBRYO SURVIVAL RATES

(57) Abstract

The present invention relates to the use of "protein painting" (the addition of exogenous, lipid–modified proteins) to improve embryonic viability, development, and implantation. It is shown here that a lipid–modified protein, e.g., GPI–modified exogenous Qa–2 protein, spontaneously incorporates into the plasma membrane of B6.K1 T cells and onto *Ped slow* preimplantation embryos. It is also shown that, given enough time after incorporation, the exogenous Qa–2 protein increases the rate of development of early embryos cultured *in vitro*, most likely by diffusing in the plasma membrane until it is "connected" to its functional signaling machinery. Protein painting of embryos has the potential, therefore, to be a valuable tool in enhancing embryo development and implantation.

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METHODS FOR ENHANCING OR REDUCING PREIMPLANTATION EMBRYO SURVIVAL RATES

RELATED APPLICATION

This application claims priority from U.S. Provisional Application No. 60/053,499, filed July 23, 1997, the whole of which is hereby incorporated by reference herein.

GOVERNMENT RIGHTS

Part of the work leading to this invention was carried out with United States Government support provided under a grant from the Department of Health and Human Services, NIH Grant No. HD31505. Therefore, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The preimplantation period of mammalian development is crucial for reproductive success. During this time the fertilized egg undergoes a series of cleavage divisions leading to the formation of a blastocyst stage embryo. In mice, domestic species, and humans, 15-50% of preimplantation embryos die during the pre-implantation period (reviewed in Clark, D.A., 1988, Janny et al., 1996). If even a small percentage of these embryos could be rescued, this would lead to the alleviation of infertility problems for many couples (as many as 3/10 of American couples are infertile) and could increase agricultural production of domestic animal species.

Data from human IVF (*in vitro* fertilization) clinics have repeatedly suggested that there are two major criteria that determine preimplantation embryo quality: (1) rate of development and (2) degree of fragmentation (e.g., Bolton et al., 1989; Levy et al., 1991; Trounson, A. and Osborn, J., 1993). Fragmentation refers to "fragments" of cells in the embryos that are smaller than normal-size blastomeres. A fast rate of development and a low degree of fragmentation

are indicative of good embryo quality. High quality embryos are more likely to give rise to live offspring than low quality embryos. Thus, methods of increasing the rate of embryo development or decreasing the degree of fragmentation are clearly desirable for enhancing embryo survival.

SUMMARY OF THE INVENTION

A study of preimplantation embryos from the mouse, domestic animals and humans has led to an understanding of the factors that cause eggs fertilized at the same time to develop at different rates. Warner and associates have discovered that the Ped (or preimplantation embryo development) gene, which maps to the Q region of the mouse major histocompatibility complex (MHC), controls the rate of cleavage of preimplantation mouse embryos and subsequent embryonic survival (Xu et al., 1994; Verbanac, K.M., and Warner, C.M., 1981; Warner et al., 1993). Specifically, these investigators have determined across various species that embryos that develop at a fast rate (*Ped fast*) have a higher chance of leading to viable offspring than embryos that develop at a slow rate (*Ped slow*). The fast *Ped* allele is the presence of the *Ped* gene product, the Qa-2 antigen encoded by the Q7 and Q9 genes, whereas the slow *Ped* allele is the absence of the Qa-2 antigen. Introduction of the Q9 gene into a *Ped slow* mouse strain was observed to convert the *Ped* gene phenotype to *fast*. The expressed Qa-2 protein is attached to the embryonic cell surface by a posttranslationally added glycosylphosphatidylinositol (GPI) anchor.

GPI-anchored proteins have recently assumed importance in protein engineering of surfaces of antigen-presenting cells without gene transfer (Tykocinski et al., PCT International Application WO 96/12009). As reviewed in Medof et al., "Cell-surface engineering with GPI-anchored proteins," Cell Surface Engineering 10:574-586, 1996, GPIs serve as membrane anchors for numerous cell-surface proteins, and isolated GPI-anchored proteins have an unusual capacity

to reintegrate with cell-surface membranes. Thus, cell surfaces can be coated, or "painted," with an externally added protein. These authors also described that a GPI moiety can be recombinantly engineered into the extracellular domain of a conventionally anchored protein (also *see* Caras et al., U.S. Patent No. 5,109,113, 1992) and that recombinant GPI-reanchored chimeric proteins can be produced in microgram-to-milligram quantities. Therefore, it was suggested that cell-surface engineering using GPI-anchored proteins provides a general means for modifying antigen-presenting cell surfaces exogenously.

The spontaneous, stable incorporation of exogenously added GPI-linked proteins into plasma membranes, also referred to as "protein painting," has been the subject of intense investigation recently because it has several advantages over standard gene transfer approaches (reviewed in Medof et al., 1996). First, GPI-linked proteins incorporate within minutes into recipient plasma membranes, even into cells that are normally difficult to transfect. Painting can be performed on a small number of cells, with nearly 100% efficiency. Additionally, unlike in gene transfer, the amount of protein incorporated into the cell surface can be easily controlled. Finally, multiple GPI-linked proteins can be painted onto the cell surface simultaneously, and many of the GPI-linked proteins that have been painted onto cells have been shown to retain their function, particularly in ligand binding (Table I below).

Table I. GPI-linked proteins that have been painted onto recipient cells

GPI-linked Protein	Normal Function	Recipient Cell	Function Maintained	Reference
DAF(CD55)	complement inhibitor	Erythrocytes	+	Medof et al., 1984
		Endothelium	+	Kooyman et al., 1995
HLA-A2.1*	antigen presentation	CRI cells	+	Huang et al., 1994
FcRIII	binds IgG for endocytosis	Jurkat cells	+	Nagarajan et al., 1995
CD4*	receptor for HIV	HeLa cells	+	Brodsky et al., 1994
CD59	complement inhibitor	Erythrocytes	+	Davies et al., 1993
				Kooyman et al., 1995
B7-1*	binds CD28	Tumor cells	+	McHugh et al., 1995
CD59	Ca2+ flux and oxidase response in neutrophils	neutrophils	-	Morgan et al., 1993
		U937 cells#	+	Van de Berg et al., 1995
Thy-1	T-cell activation	T-cells	-	Zhang et al., 1992

[a]The exogenous GPI-protein was compared to the endogenous GPI-linked protein in terms of function. (+) = function was maintained; (-) = function was not maintained.

* = Genetically engineered to be GPI-linked to the cell surface.

= Function studied two hours after incorporation of CD59.

The methods of the invention bring the results of the above-described research to bear on the problem of embryo survival. It is suggested and shown herein that the Qa-2 protein, or other appropriate protein, can be "painted" on the surface of oocytes or preimplantation embryos. The proteins enter the cell membrane because they contain a lipid, preferably a GPI, "tail" (Fig. 1). Fig. 1 shows a schematic of protein painting of exogenous Qa-2 protein onto recipient cells. The lipid molecules in the GPI tail spontaneously, but stably, incorporate into the outer leaflet of the lipid bilayer of the recipient cell membranes.

Depending on the particular protein chosen for the painting procedure, preimplantation embryonic survival could either be enhanced or reduced. It is advantageous to use protein painting to alter oocytes or embryos because, unlike genetic engineering with DNA, engineering with proteins is transient and should have no long-lasting effects on the offspring. Painting *e.g.*, Qa-2 protein, onto the surface of *Ped slow* preimplantation embryos would be a practical alternative to the difficult and tedious procedure of microinjection of the *Ped* gene(s) involved in embryonic cleavage and survival.

Examples are given that show that partially purified Qa-2 protein (the *Ped* gene product) can be painted onto the surface of T cells and preimplantation embryos *in vitro*, and that the exogenous Qa-2 protein can increase embryonic cleavage rate after equilibration on the cell surface enhancing the likelihood of embryonic survival.

Enhanced embryonic survival by treatment with an exogenously applied, species-appropriate equivalent to Qa-2 (e.g. HLA-E or HLA-G), would have immediate application for human *in vitro* fertilization (IVF) clinics, where only 20-30% of embryos fertilized *in vitro* survive to term. There is also potential therapeutic use for domestic animal species. For instance, increasing the number of pigs per litter is of

economic importance to pork producers in the billions of dollars.

Alternatively, as genes involved in apoptosis (programmed cell death) appear to control the degree of fragmentation of embryos, a GPI-linked "tail" could be attached to an appropriate apoptosis-related gene product and be used either to prevent or increase fragmentation, depending on whether the desired outcome was increased embryo survival or embryo death. To decrease fragmentation one could paint on gene products that suppress apoptosis, such as anti-apoptotic members of the Bcl-2 family. The surface-modified oocyte would then be fertilized and allowed to develop. The surface-modified embryo would be cultured in a growth-promoting environment having conditions that promote the implantation, the rate of development, and the probability of survival of the embryo after its transfer to a recipient mother.

In addition, a GPI-linked "tail" could be engineered onto a protein that would recognize and bind only to an embryo (as opposed to maternal tissue), to cause embryonic demise. A composition containing such a construct could be incorporated into a vaginal suppository to provide a new method of contraception.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts a schematic of protein painting of exogenous Qa-2 protein onto recipient cells.

Fig. 2 depicts a FACScan analysis of various titrations of B6.K2 lymphocyte lysate on B6.K1 ($Qa\text{-}2^-$) T cells for incorporation of Qa-2 protein;

Fig. 3 depicts a FACScan analysis of various culture conditions used to incorporate exogenous Qa-2 protein onto B6.K1 T cells;

Fig. 4 depicts bar graphs showing the effect of Qa-2 crosslinking on the proliferative responses of B6.K1 T cells incubated with various concentrations of B6.K2 lymphocyte

lysate (Fig. 4A) and, as a control, of B6.K2 lymphocytes incubated with various concentrations B6.K1 lysate (Fig. 4B);

Fig. 5 depicts a bar graph showing the effect of Qa-2 cross-linking on the proliferative responses of B6.K1 T cells incubated with various concentrations of B6.K2 lymphocyte lysate for 0, 2, or 8 hours or with PHA as a control; and

Fig. 6 depicts I-PCR analysis of B6.K1 preimplantation embryos painted with exogenous Qa-2 protein containing lysate.

DETAILED DESCRIPTION OF THE INVENTION

In the following section, protocols are given for the preparation and use of the Qa-2 antigen merely as an exemplary protein for use in the methods of the invention. The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in the art in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure. In particular, it is understood that other proteins that affect embryonic survival in various species, including humans and domestic animals, may be used.

GPI-linked Qa-2 antigen can be purified, e.g., from T lymphocytes from C57BL/6 mice or from the murine thymoma cell line, R1.1, which overexpresses class I molecules. The purified Qa-2 can then be painted onto Qa-2 negative ($Qa\text{-}2^-$) T lymphocytes from B6.K1 mice (as test cells). The painted protein is checked for stability, the GPI-anchor is confirmed, and the functionality of the anchored protein is determined by Qa-2-mediated crosslinking. After the painted Qa-2 is established as being functional, it is painted onto B6.K1 ($Qa\text{-}2^-$) preimplantation embryos at different stages of development. The embryos are checked for a change in phenotype from *Ped slow* to *Ped fast* by quantitating the number of cells per embryo.

After the viability of external application of the mouse Qa-2 antigen is tested and optimized in the mouse system,

a similar protocol can be used to paint a Qa-2 homolog onto embryos in another mammalian system. For instance, the GPI "tail" is transferred from the mouse protein onto a human or domestic animal equivalent to Qa-2 (e.g., HLA-G would be a suitable human protein), and the synthetic construct is used for protein painting. This method would be useful therapeutically to achieve pregnancies in women who formerly could not become pregnant because their embryos were developing too slowly. Use of the heterologous construct -- e.g., human HLA-G with the mouse Qa-2 GPI "tail"-- is advantageous compared to using the intact mouse Qa-2 protein, in terms of preventing or minimizing the risk of unwanted immunological reactions of the human maternal immune system against the treated embryo.

A. Purification of Qa-2 from mouse T lymphocytes or R1.1 cells

One may use the following protocol to prepare Qa-2 in quantity, which is based on that described by Nagarajan et al., "Purification and optimization of functional reconstitution on the surface of leukemic cell lines of GPI-anchored FcR III," (Nagarajan et al., 1995). Cells are cultured in medium comprising: RPMI 1640 + 10% fetal calf serum (FCS) + antibiotics + 5 mM glutamine. Nylon wool-enriched T cells are obtained from C57BL/6 mouse spleens in the same medium. Cells (approximately $1x10^8$ total) are lysed in lysis buffer (comprising 50 mM Tris-HCl pH 8.0, 1% Triton X-100, 5 mM iodoacetamide, 1 mM PMSF, 1% aprotinin, and 2 mM diisopropyl fluorophosphate) for 1 hour at 4° C. The lysate is then centrifuged at 10,000 x g for 1 hour. The supernatant, containing Qa-2, is passed over a CNBr-activated Sepharose (Pierce) affinity column coupled to anti-Qa-2 monoclonal antibodies. Anti-Qa-2 monoclonal antibodies are commercially available (e.g., 1-1-2 (mouse IgGa), Cat. No. 06321D and 1-9-9 (mouse IgG2a), Cat. No. 06331D, Pharmingen (San Diego, CA); 1416.6 (mouse IgG2b), Cat. No. MCA1012, Serotec (Washington, D.C.)).

After extensive washing of the column, Qa-2 is eluted with 50 mM glycine-HCl pH 3.0/1% Triton X-100. One-ml fractions are collected and neutralized with 1 M Tris-HCl pH 10. Following the purification procedure, Qa-2 can be subjected to SDS-PAGE and Western blot analysis to determine purity and specificity.

B. Partial purification of Qa-2 protein from B6.K2 splenocytes

One of the original studies demonstrating that proteins could be painted onto the surface of recipient cells did not use purified protein as the paint. Instead, partially purified membrane preparations were incubated with erythrocytes, and it was found that DAF (CD55) selectively incorporated into the erythrocyte membranes and was functional in protecting the cells from complement-mediated lysis (Medof et al., 1984). Therefore, we partially purified Qa-2 from B6.K2 splenocytes, concentrated the lysate with simultaneous detergent removal, and incubated the lysate with B6.K1 T cells, as discussed below.

Two spleens from either B6.K2 ($Qa\text{-}2^+$) mice or B6.K1 ($Qa\text{-}2^-$) mice were rubbed over a wire mesh screen and rinsed with phosphate-buffered saline(PBS) to create a cell suspension, as described previously. The cell suspension was enriched for lymphocytes by Ficoll-Hypaque density gradient centrifugation, and the lymphocyte layer was rinsed twice in PBS. The density was adjusted to about $5x10^7$ cells/ml in lysis buffer (comprised of 50 mM NaCl + 2% NP-40), and 200 μl of a mammalian protease-inhibitor cocktail (Sigma) were added. The cells were vortexed vigorously for several seconds, and were placed on ice for one to two hours, with intermittent vortexing to ensure lysis. At this point, the suspension was centrifuged at 1500xg for 10 minutes at 4°C, and the pellet was discarded. The supernatant was centrifuged at 10,000xg for an additional 15 minutes, and was frozen at -20°C until it could be concentrated. For concentration of the lysate with simultaneous detergent

removal, 2 ml of the lysate were placed into a Centricon-10 and centrifuged at 5000xg for one hour (Millipore). The centrifugation step was repeated 5 times, and the retentate was diluted with PBS to a final volume of 2 ml with each spin, in order to ensure complete removal of the detergent. The final concentrated retentate was aliquoted and kept at -20°C until use, at which point it was diluted in PBS and titered on Qa-2 negative (Qa-2$^-$) T-cells by FACScan analysis, as discussed in Example I below.

C. Protocol for protein painting of Qa-2

All conditions are optimized for incorporation of Qa-2 into the cell membrane of lymphocytes (as test cells) and embryos, including time of incubation, detergent concentration, temperature, amount of Qa-2, and whether or not embryos need to have the zona pellucida removed. As a starting point, $1x10^6$ cells/ml of B6.K1 T lymphocytes (Qa-2 negative) are washed three times in PBS to remove residual fetal calf serum (FCS), and placed into flow cytometry tubes. Approximately 100-200 ng of Qa-2 are then added to the cells. Following incubation for 1-2 hours at 37°C, the cells are washed three times in PBS and subjected to FACScan analysis to confirm attachment of the Qa-2 to the recipient cell membrane. Crosslinking studies, using a protocol previously established for Qa-2-mediated T cell activation (Robinson et al., 1989), are then carried out to establish that the Qa-2 antigen not only has been incorporated into the cell membrane, but also is functional. The optimized conditions determined for painting Qa-2 protein onto T cells are then used as a starting point to determine the optimum conditions for painting Qa-2 onto preimplantation mouse embryos.

Example I: Painting of B6.K1 T-cells with Qa-2 protein from partially purified B6.K2 lysate

Qa-2 protein was first painted onto the surface of T lymphocytes, to determine optimal conditions for painting

Qa-2 onto embryos of the *Ped slow* phenotype, *e.g.*, B6.K1 mice lacking Qa-2 protein expression.

Nylon wool-enriched T-cells were obtained from B6.K1 mice and B6.K2 mice (as the Qa-2$^+$ control) and washed three times in PBS in order to remove residual fetal calf serum (FCS). The cells were resuspended at a density of $5x10^6$ cells/ml in PBS, and 100 μl of the cell suspension were added to FACScan tubes (Falcon) for a final concentration of $5x10^5$ cells/tube. Fifty (50) μl of the partially purified B6.K2 splenocyte lysate suspended in PBS (at dilutions ranging from 1:2 to 1:2000) were added to designated tubes, while the B6.K1 lysate was added to control tubes. The cells were incubated at 37°C, 5% CO_2 for one hour, and then washed three times in PBS + 1% BSA + 0.01% azide. At this point, FACScan analysis was performed, using the Qa-2-specific monoclonal antibody, 1-12-1, at a final concentration of 5 μg/ml, to determine whether the B6.K1 cells had incorporated Qa-2 protein from the B6.K2 lysate.

Titration of the partially purified B6.K2 lymphocyte lysate on B6.K1 T-cells.

Qa-2 was partially purified from a B6.K2 lymphocyte lysate, and the lysate was incubated at various dilutions with $5x10^5$ B6.K1 T-cells suspended in PBS. Fig. 2 is a FACScan analysis of the B6.K1 T-cells after addition of B6.K2 lysate containing exogenous Qa-2 protein, or addition of lysate from B6.K1 T-cells as a control. B6.K1 T-cells suspended in PBS (at a concentration of about $5x10^5$ cells/FACScan tube) were incubated with: (i) 50 μl of PBS only (peak 1), to detect autofluorescence; (ii) 50 μl of various dilutions of B6.K1 lymphocyte lysate (peak), to detect non-specific binding; or (iii) 50 μl of various dilutions of B6.K2 lymphocyte lysate (peak 3), to detect incorporation of exogenous Qa-2 protein. After a one-hour incubation at 37°C, the cells were washed in PBS, and Qa-2 protein was detected by immunostaining with 1-12-1 mAb. In

Fig. 2, Panel A shows incubation with undiluted lysate; Panel B shows incubation with 1:2 dilution of lysate; Panel C shows incubation with 1:10 dilution of lysate; Panel D shows incubation with 1:100 dilution of lysate; and Panel E shows incubation with 1:1000 dilution of lysate.

The FACScan results in Fig. 2 show that Qa-2 protein does incorporate into the cell membrane of B6.K1 T-lymphocytes in a concentration-dependent manner, with optimal Qa-2 incorporation occurring when using B6.K2 lysate dilutions of approximately 1:2, 1:10, and 1:100.

EXAMPLE II: Optimal conditions for painting Qa-2 protein onto B6.K1 T-cells

It has previously been shown that the incorporation of GPI-linked proteins is inhibited by bovine serum albumin (BSA) and by low temperatures (reviewed in Medof, 1996). Therefore, the optimal conditions for incorporation of exogenous Qa-2 protein onto the surface of B6.K1 T-cells were determined (as a starting point for determining the optimal conditions for painting Qa-2 onto preimplantation embryos). It was determined that painting of the Qa-2 protein onto the cell surface was inhibited by BSA and by low temperature (as shown in Fig. 3), two hallmark characteristics of GPI-linked protein incorporation (reviewed in Ilangumanran et al., 1996; Medof et al., 1996). The BSA likely interferes with the GPI anchor's interaction with lipids in the plasma membrane, while the low temperature is thought to affect membrane fluidity dynamics that may be required for GPI's successful incorporation.

Fig. 3 shows the FACScan analysis of optimal conditions for incorporation of exogenous Qa-2 protein on B6.K1 T-cells. B6.K1 T-cells ($5x10^5$ cells/FACScan tube) were incubated for 1 hour with: (i) 50 μl of a 1:100 dilution, or otherwise noted dilutions, of B6.K2 lymphocyte lysate (peak 2); or (ii) PBS only (peak 1). Panel A shows the results of a 1:5000 dilution of lysate incubated at 37°C; Panel B shows a 1:100 dilution of lysate at 37°C; Panel C shows a 1:100 dilution of lysate at 4°C; Panel D shows a 1:100 dilution of

lysate diluted in PBS+1%BSA, incubated at 37°C; Panel E shows a 1:10 dilution of B6.K1 lysate incubated at 37°C; Panel F shows a 1:100 dilution of B6.K1 lysate incubated at 37°C.

Exogenous Qa-2 protein from B6.K2 lysates incorporated into the plasma membranes of B6.K1 T-cells after a one-hour incubation at 37°C, when the T-cells and lysate were diluted in PBS (Fig. 3, panel B). However, this incorporation was inhibited when the T-cells were incubated with the lysate at 4°C (Fig. 3, panel C), or when the T-cells and lysate were diluted in PBS + 1%BSA (PBSA) (Fig. 3, panel D), or when the lysate was diluted in PBS at low concentrations (1:5000, panel A). B6.K1 T-cells incubated with B6.K1 lysate (the control) did not incorporate Qa-2 protein, as expected (panels E and F).

FACScan results on painted B6.K1 T cells (Figs. 2 and 3) show that, at optimal dilutions of the B6.K2 lymphocyte lysate, the levels of incorporated Qa-2 protein appear to be at least equivalent to, if not greater than, the amount of endogenous Qa-2 protein on the surface of C57BL/6 and B6.K2 T cells. One of ordinary skill in the art will appreciate that further analysis by quantitative I-PCR can be used to determine: the threshold and/or optimal level of Qa-2 or its homolog needed to increase the rate of development of *Ped slow* embryos, and the length of time that exogenously added Qa-2 remains on the embryo's surface.

Similar results are expected with preimplantation embryos.

Example III: Crosslinking of the Qa-2 protein painted onto B6.K1 cells

To determine whether the exogenous Qa-2 protein that was incorporated into the plasma membrane of B6.K1 T-cells was functional, the exogenously added Qa-2 protein was crosslinked to determine if those cells would undergo T-cell activation. The same conditions were used as for cell isolation, in terms of media, solution concentrations, incubation times, and lysate concentrations (discussed later

with reference to Fig. 4), except that the cells were washed in RPMI 1640 medium after incubation with the partially purified Qa-2 protein.

After incubating the B6.K1 T-cells in either a 1:10 or 1:100 dilution of the partially purified B6.K2 lymphocyte lysate, the cells were then incubated with 1-12-1 anti-Qa-2 monoclonal antibody (mAb) and the exogenous Qa-2 protein cross-linked in the presence of PMA, to determine if the B6.K1 T-cells would undergo activation. B6.K2 T-cells alone, or B6.K2 T-cells incubated with a 1:10 or 1:100 dilution of B6.K1 lymphocyte lysate, were included as positive controls.

Fig. 4 depicts the results of crosslinking the exogenous Qa-2 protein painted onto B6.K1 T-cells. Specifically, B6.K1 T-cells ($5x10^5$ cells/well) were incubated with either (i) 50 μl of PBS only (control), or (ii) a 1:10 or 1:100 dilution of B6.K2 lymphocyte lysate in PBS, at 37°C for one hour, and washed three times in RPMI 1640 complete medium. For a control, B6.K2 T-cells were incubated under the same conditions in PBS only, or in a 1:10 or 1:00 dilution of B6.K1 lymphocyte lysate (lacking Qa-2 protein). The T-cells were then incubated with 50 μg/ml of 1-12-1 anti-Qa-2 mAb, for 30 minutes at room temperature, and then cultured for 48 hours in second antibody and PMA. One μCi of [^{3}H-TdR] was added for the last 6 hours of culture, and the cells were harvested. Results are from one experiment, performed in triplicate, and presented as mean cpm ± SEM.

Fig. 4A (top panel) shows that B6.K1 T-cells incubated with the B6.K2 lysate for 1 hour could not be activated by crosslinking with an Ab and PMA. The B6.K2 T-cells incubated in the B6.K1 cell lysate, as a control, were activated (Fig. 4B bottom panel). These results suggested that a longer time of incubation was needed for the exogenously added Qa-2 protein to become functional after incorporation onto the painted cells.

Example IV: Effect of allowing "equilibration" of the Qa-2 protein painted onto B6.K1 cells

It has been proposed that other factors, namely, the association of GPI-linked proteins with accessory molecules, is required for the exogenous GPI-linked protein's full signaling function (Morgan et al., 1993; van de Berg et al., 1995). To address this issue, another crosslinking study was performed where the exogenously added Qa-2 protein was allowed to "equilibrate" in the plasma membrane before being crosslinked. It was found that the painted Qa-2 protein did not allow signaling of T cells until it was allowed to equilibrate, or diffuse in the membrane, for 8 hours, and that only the T cells incubated with a higher concentration of Qa-2 protein (a 1:10 dilution of lysate) were able to respond to crosslinking of Qa-2, suggesting that the density of Qa-2 protein is crucial for signaling (Fig. 5).

The fact that an equilibration period (before crosslinking of the exogenous Qa-2 protein) was required to elicit an activation response agrees with results from another crosslinking study. Exogenously added CD59 initially localized in a diffuse manner in the plasma membrane of recipient cells, and was unable to induce cell activation immediately after crosslinking. However, after an equilibration period on the cell surface, the exogenous CD59 clustered into high MW kinase-containing complexes, and crosslinking of the protein resulted in signal transduction comparable in magnitude to crosslinking of the endogenous CD59 (Van de Berg, 1995). These results, along with our crosslinking results, suggest that exogenously added GPI-linked proteins can become functional in terms of their signaling capacity, in a time dependent manner.

To determine if the exogenous Qa-2 protein would become functional after "equilibration" in the plasma membrane, painted B6.K1 T-cells were first incubated for either 0, 2, or 8 hours after incorporation of Qa-2 protein, and then treated with 1-2-1 mAb and crosslinking solution, or with PHA.

Fig. 5 shows the effect on T cell activation, via crosslinking of exogenous Qa-2 protein painted onto B6.K1 T-cells, after allowing equilibration of the Qa-2 in the plasma membrane. B6.K1 T-cells were incubated with a 1:10 or 1:100 dilution of B6.K2 lymphocyte lysate for Qa-2 protein painting. After incubation, the cells were washed and resuspended in RPMI 1640 medium, and placed into the wells of a 96-well microtiter plate (time 0). After 0, 2, or 8 hours in culture to allow Qa-2 equilibration, the cells were incubated with a 1:100 dilution of PHA, or were incubated with 1-12-1 mAb for 30 minutes and cultured in crosslinking solution, as previously described. After 42 hours in culture, 1 μCi of [^{3}H-TdR] was added to each well, and the cells were cultured for an additional 6 hours before harvesting. The results are from one experiment and are presented as mean cpm from duplicate wells ± SEM.

Fig. 5 shows that the Qa-2-painted T-cells were activated by PHA at all the timepoints assayed. The painted T-cells did not respond to crosslinking of the exogenous Qa-2 protein immediately after incorporation (time = 0 hours) or after a 2-hour equilibration period. However, after approximately 8 hours of equilibration, the T-cells that had been incubated with a 1:10 dilution of the B6.K2 lymphocyte lysate were activated at a low level after crosslinking of the exogenous Qa-2 protein.

One of ordinary skill in the art will also be able to determine, in light of this specification, an optimal Qa-2 concentration and an optimal incubation time for allowing this *Ped* gene product (or its analog in other mammals) to equilibrate in the plasma membrane of recipient cells and preimplantation embryos.

D. Protocol for Using Protein Painting to Enhance Embryo Survival

A mouse embryo test system is used to develop appropriate conditions for use in the desired end-product species. For example, one may paint Qa-2 onto B6.K1 mouse embryos, ranging from the two-cell to the blastocyst (32-cell) stage of development, and assess their conversion from the *Ped slow* to the *Ped fast* phenotype. Alternatively, protein painting maybe performed on oocytes (unfertilized eggs). Various concentrations of Qa-2 protein, ranging from the attomolar to the millimolar range, may be tested and the optimal concentration chosen that converts the phenotype of the mouse oocytes or embryos from *Ped slow* to *Ped fast*.

Using the optimal conditions for painting of Qa-2 protein onto T cells, *Ped slow* embryos were also painted. It was found that Qa2 protein incorporated into the membranes of *Ped slow* 8-cell embryos, as determined by Immuno-PCR (I-PCR) analysis (Fig. 6, discussed later in Example V). To determine functionality, the exogenous Qa-2 protein was added to *Ped slow* embryos and allowed to equilibrate in the plasma membrane for 24 or 48 hours, at which point the number of cells per embryo was quantified. It was found that the addition of exogenous Qa-2 protein to embryos had no effect on their rate of cleavage after 24 hours in culture, compared to control embryos that were not painted with Qa-2 protein (Group I, Table II). However, *Ped slow* embryos that were painted with exogenous Qa-2 protein and cultured for 48 hours did increase their rate of cleavage, compared to control embryos that were not painted (Group II, Table II). Therefore, the exogenous Qa-2 protein is able to retain at least part of its function in preimplantation embryonic development, if it is allowed to equilibrate on the embryonic surface for a sufficient amount of time.

EXAMPLE V: Painting of Qa-2 onto *Ped slow* preimplantation embryos

CBA/Ca (Qa-2-) or B6.K1(Qa-2-) 8-cell embryos were collected into Whitten-Biggers (WB) medium, pooled, and washed three times in WB without BSA. The embryos were then incubated with the partially purified lysate from B6.K1 splenocytes (Qa-2$^-$control) or B6.K2 (Qa-2$^+$) splenocytes, at dilutions ranging from 1:100 to 1:200 (in WB without BSA), for 45 minutes at room temperature to minimize damage from any residual detergent. The embryos were then washed three times in WB with BSA, and either cultured in microdrops under oil for 24 hours or 48 hours, or placed into PBSAZ for I-PCR analysis. For embryos in culture, the number of cells per embryo was quantified after 24 hours or 48 hours, by staining with bisbenzamide. (The embryos were incubated with 5 μg/ml bisbenzamide in WB medium for 30 minutes at 37°C).

Fig. 6 shows the I-PCR analysis of B6.K1 preimplantation embryos painted with exogenous Qa-2 protein. B6.K1 8-cell embryos were incubated in a 1:100 dilution of B6.K2 lymphocyte lysate containing Qa-2 protein, or in a 1:100 dilution of B6.K1 lymphocyte lysage (control), in WB medium without BSA, for one hour at room temperature. The embryos were then washed three times in PBSAZ, and the zona pellucidae were removed by a 3-5 minute incubation in Acid Tyrode's solution (pH 2.5). After an additional three washes in PBSAZ, the embryos were incubated for one hour in 0.5 mg/ml of 1-12-1 mAb at 4°C, washed extensively, and then incubated in $1x10^{-15}$ M chimeric protein-DNA complex for 45 minutes, at 4°C. PCR was then carried out. In Fig. 6 the lanes show the following: Lane 1: Blank; Lane 2: 5 embryos + B6.K1 lysate; Lane 3: 3 embryos + B6.K1 lysate; Lane 4: 2 embryos + B6.K1 lysate; Lane 5: 3 embryos + B6.K2 lysate; Lanes 6-8: single embryos + B6.K2 lysate; Lane 9: Chimeric protein - DNA complex (positive control); Lane 10: PBSAZ (negative control).

Fig. 6 demonstrates that after a one-hour incubation in a 1:100 dilution of the B6.K2 lymphocyte lysate, Qa-2 protein

was detectable on the surface of B6.K1 8-cell embryos (lanes 5-8). Embryos incubated with a 1:100 dilution of lysate from B6.K1 lymphocytes did not incorporate Qa-2 protein, as expected (lanes 1-4). The results show that preimplantation embryos, like T-cells, can be painted with GPI-linked Qa-2 protein.

Example VI: Quantitation of the total number of cells in *Ped slow* embryos painted with Qa-2 protein

In order to determine if *Ped slow* embryos increase their rate of cleavage after the addition of exogenous Qa-2 protein, 8-cell embryos from the CBA/Ca(Qa-2$^-$) or B6.K1(Qa-2$^-$) mouse strains were incubated in partially purified Qa-2 protein and cultured for either 24 hours or 48 hours. The number of cells per embryo was determined as shown in Table II below. Embryos incubated for 24 hours did not have significantly higher numbers of cells per embryo compared to control embryos. However, after 48 hours in culture, embryos incubated in a 1:200 dilution of the B6.K2 splenocyte lysate exhibited a significant increase ($P<0.05$) in cleavage rate, compared to the control embryos. Embryos painted with a 1:100 dilution of the B6.K2 splenocyte lysate also had increased cleavage rates after 48 hours, but the P value was not significant at the 0.05 level ($P = 0.11$).

It is not known why exogenous Qa-2 protein did not affect the cleavage rate of 8-cell embryos cultured for 24 hours. The removal of Qa-2 protein by PI-PLC has been shown to slow preimplantation embryonic development after 24 hours in culture (Tian et al., 1991), so one would expect that the addition of exogenous Qa-2 protein on the surface of embryos should increase development after 24 hours. It is possible that other proteins in the partially purified B6.K2 lymphocyte lysate were painted onto the embryonic surface along with Qa-2, which subsequently affected Qa-2 protein's initial signaling capabilities. Using substantially purified Qa-2 protein in the method of the invention should increase the development rate at least as much or even more than what

was observed by using partially purified Qa-2. It is also possible that Qa-2 could increase preimplantation embryo development more substantially if it is painted onto oocytes or onto embryos at earlier stages of development, such as the 2-cell stage.

Table II. Quantitation of the number of blastomeres in *Ped slow* 8-cell embryos painted with exogenous Qa-2 protein and cultured for 24 or 48 hours.

# exp.	n	Concentration of Qa-2$^+$ lysate[a]			
		no lysate	1:100 K1	1:100 K2	1:200 K2
Group I (24 hours in culture)					
2	219	17.4±4.5	17.1±2.9	17.9±4.1	17.5±4.7
Group II (48 hours in culture)					
1	118	20.9±5.7	20.9±6.5	23.4±5.6 #	25.5±6.4 *

Above results are shown as mean number of blastomeres ± SEM

[a]K1 = lysate from B6.K1 splenocytes, used as a control.

K2 = lysate from B6.K2 splenocytes, used for painting of Qa-2 protein.

* = Significantly different from control embryos painted with 1:100 dilution of B6.K1 lysate at the $P < 0.05$ level.

= Significantly different from control embryos painted with 1:100 dilution of B6.K1 lysate at the $P = 0.11$ level.

E. Protocol to Enhance Survival of Human Embryos for IVF

It is standard practice in IVF clinics to use a mouse monitoring system to test protocols for extension to the human system. For extension of the method of the invention to the human system, spare human ooctyes or embryos from an IVF clinic are treated with an appropriate concentration of a selected GPI-linked protein, ranging from the attomolar to millimolar range, as determined from the mouse system. The fertilized human eggs are incubated for 1-5 days. One then

assesses the rate of development with and without added GPI-linked protein to verify that treatment with the GPI-linked protein increases the rate of development of the embryos. The treated oocytes or embryos should have a greater chance of resulting in live offspring, after surgical transfer back to the mothers, than the untreated oocytes or embryos.

F. Determination of the minimum amount of GPI-anchor necessary for Qa-2 function

It has been shown that the terminal 36 amino acids of another GPI-linked molecule, DAF, was necessary to allow a chimera of the DAF tail and the HLA-A2.1 molecule (a similar molecule to Qa-2) to attach to the cell surface. A longer tail of DAF of 89 amino acids was not functional (Brunschwig et al., 1995). For Qa-2, it has been shown that all of the signals required for GPI-linkage to the cell membranes are confined to the 36 amino acids in the carboxy-terminal tail of the molecule (Ulker et al., 1990). Qa-2 antigen is encoded by the Q6, Q7, Q8 and Q9 genes, which contain 8 exons. GPI-anchored Qa-2 has a signal in exon 5 for the GPI "tail." As an example, the Q9 gene has 117 nucleotides in exon 5, and there are 18 sites for known restriction enzymes. It would therefore be possible to cut exon 5 with different restriction enzymes, using routine procedures, to create varying lengths of the Qa-2 GPI anchor signal sequence and engineer different DNA constructs that could produce Qa-2 with tails of variable lengths to test for functionality. Alternatively, PCR products of any length could be generated by using appropriate primers based on the known sequence of the Q9 gene (Cai et al., 1996). After the constructs were generated, they would be cloned and transfected into a mammalian cell line to generate a large amount of the modified Qa-2 protein. Cell lysates from the transfected cells could then be purified by immunoaffinity chromatography and painted onto tester cells. Function would be assessed by crosslinking the Qa-2 antigen on the tester cells with anti-Qa-2 monoclonal antibody. Functional GPI-linked Qa-2 protein will result in cell proliferation, which can be

measured by incorporation of a radioactively labeled isotope into DNA.

G. Other uses of protein painting to control embryo development

In human *in vitro* fertilization (IVF) clinics, there are two criteria that determine the chances that a particular embryo will lead to a successful pregnancy: (1) rate of development (the *Ped* gene effect) and (2) degree of fragmentation. The faster the rate of development and the lower the degree of fragmentation the more likely that a given embryo will give rise to a baby. As described above, the GPI anchor from the Qa-2 molecule can be added to "Ped-like" molecules, such as HLA-G (i.e., members of the class I major histocompatibility complex family of molecules), to remedy the first problem with embryonic viability, slow rate of development.

A similar approach would be used to remedy the second problem of embryonic viability, too much fragmentation. In women who tend to produce highly fragmented embryos, proteins that inhibit apoptosis (programmed cell death) could be painted on the embryonic cell surface early in development, in an effort to inhibit fragmentation. The candidates for protein painting to inhibit apoptosis would be members of the Bcl-2 family.

Another use of this technology would be to enhance implantation of the embryo in the uterus. For instance, a member of the adhesion family of proteins could be painted on the embryos to enhance "sticking," or implantation, to the uterus lining.

Furthermore, suitable proteins could be painted on embryos to prevent rejection of the embryos by the maternal immune system, a phenomenon thought to occur in some cases of spontaneous abortion. Two possible candidate proteins for such a use are HLA-G and DAF, both of which may be immunoprotective molecules.

The protein painting procedure as described herein could also be used to selectively target embryos with proteins that

enhance cell death, thereby providing a novel contraceptive technique. Some members of the Bcl-2 family enhance cell death via apoptotic mechanisms and a second gene family, the caspase family of proteins, also mediate cell death via apoptotic mechanisms. In addition, members of the TNF superfamily may also be candidates to mediate the death of the embryo. Reagents would be designed that specifically target the embryos and not the maternal tissues. One approach is to put the GPI-linked proteins into liposomes or other carriers that could be made to recognize only embryos and not the maternal tissues. For example, a liposome or carrier could be designed to specifically recognize zona pellucida proteins that are found only on embryos and not on maternal tissues, thereby allowing the chosen GPI-linked protein to attach to and kill only the embryos.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein.

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CLAIMS

What is claimed:

1. A method for increasing the rate of development of an embryo comprising the steps of:

contacting the external portion of an embryo at a preimplantation stage with an externally applied, lipid-modified MHC polypeptide;

culturing said modified embryo in a growth-promoting environment having conditions that promote the implantation, the rate of development, and the probability of survival of said embryo; and

transferring said embryo to a recipient mother.

2. The method of claim 1, wherein said preimplantation stage is within the range spanning from the two-cell to the blastocyst stage.

3. A method for increasing the rate of development of an embryo comprising the steps of:

contacting the external portion of an oocyte with an externally applied, lipid-modified MHC polypeptide;

fertilizing said modified oocyte;

culturing said fertilized oocyte in a growth-promoting environment having conditions that promote the implantation, the rate of development, and the probability of survival of the resulting embryo; and

transferring said embryo to a recipient mother.

4. The method of claim 1 or 3, wherein said lipid is glycosyl-phosphatidylinositol (GPI).

5. The method of claim 1 or 3, wherein said MHC polypeptide is the product of the mouse *Ped* gene or of the homologous gene in another mammal.

6. The method of claim 1 or 3, wherein said MHC polypeptide is Qa-2, HLA-E, or HLA-G.

7. A method for affecting the degree of fragmentation of an embryo comprising the step of:

contacting the external portion of an embryo at a preimplantation stage with an externally applied, lipid-modified, apoptosis-related gene product.

8. The method of claim 7, used to decrease fragmentation, further comprising a step of:

culturing said modified embryo in a growth-promoting environment having conditions that promote the implantation, the rate of development, and the probability of survival of said embryo; and

transferring said embryo to a recipient mother.

9. A method for decreasing the fragmentation of an embryo, comprising the step of:

contacting the external portion of an oocyte with an externally applied, lipid-modified, apoptosis-related gene product;

fertilizing said modified oocyte;

culturing said fertilized oocyte in a growth-promoting environment having conditions that promote the implantation, the rate of development, and the probability of survival of the resulting embryo; and

transferring said embryo to a recipient mother.

10. The method of claim 8 or 9, wherein said apoptosis-related gene product suppresses apoptosis.

11. The method of claim 10, wherein said apoptosis-related gene product is an anti-apoptotic member of the Bcl-2 family.

12. The method of claim 7, for increasing fragmentation, wherein said apoptosis-related gene product promotes apoptosis.

13. The method of claim 12, wherein said apoptosis-related gene product is a pro-apoptotic member of the Bcl-2 family or the caspase family.

14. The method of claim 12, wherein said apoptosis-related gene product is Bax.

15. A method for enhancing implantation of an embryo comprising the steps of:

contacting the external portion of an embryo at a preimplantation stage with an externally applied, lipid-modified adhesion molecule;

culturing said modified embryo in an environment having conditions that promote the implantation, the rate of development, and the probability of survival of said embryo; and

transferring said embryo to a recipient mother.

16. The method of claim 15, wherein said lipid is glycosyl-phosphatidylinositol (GPI).

17. A method of reducing the risk of rejection of an embryo by the maternal immue system, comprising the steps of:

contacting the external portion of an embryo at a preimplantation stage with an externally applied, lipid-modified immunoprotective molecule;

culturing said modified embryo in an environment having conditions that promote the implantation, the rate of development, and the probability of survival of said embryo; and

transferring said embryo to a recipient mother.

18. The method of claim 17, wherein said immunoprotective molecule is HLA-E, HLA-G or decay-accelerating factor (DAF).

19. A composition comprising a vaginal suppository and a lipid-modified protein that promotes apoptosis or increases embryonic fragmentation.

20. The composition of claim 19, wherein said lipid is glycosylphosphatidylinositol (GPI).

21. The composition of claim 19, wherein said protein is a pro-apoptic member of the Bcl-2 family or the caspase family.

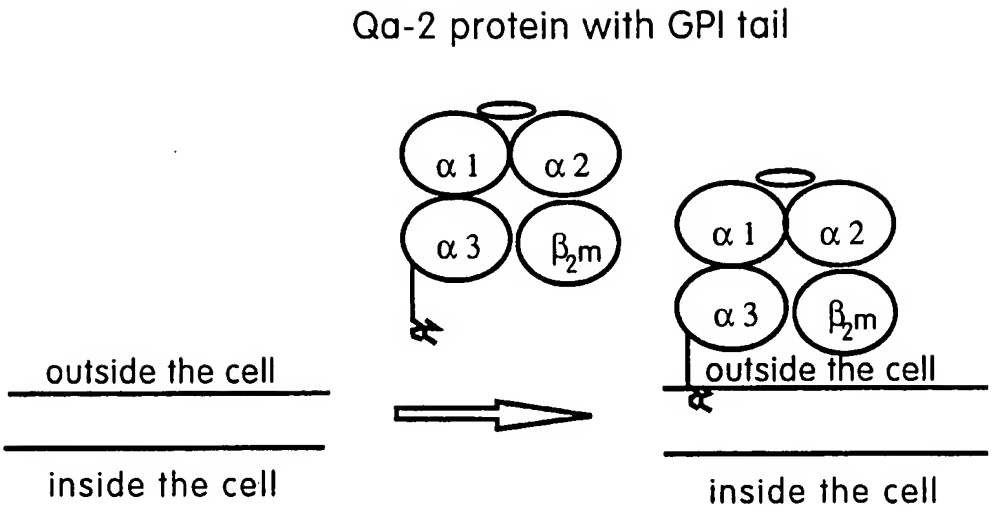

Qa-2 protein with GPI tail
α 1
α 2
α 3
β2m
outside the cell
inside the cell
α 1
α 2
α 3
β2m
outside the cell
inside the cell

Fig. 1

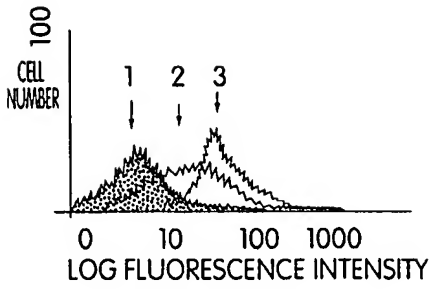


Fig. 2A

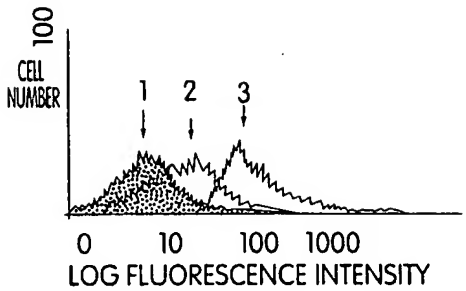


Fig. 2B

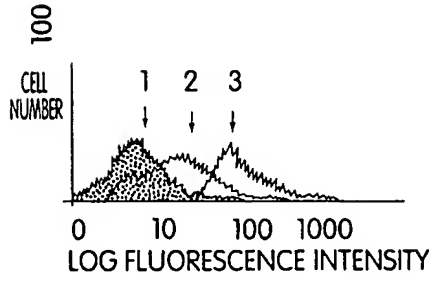


Fig. 2C

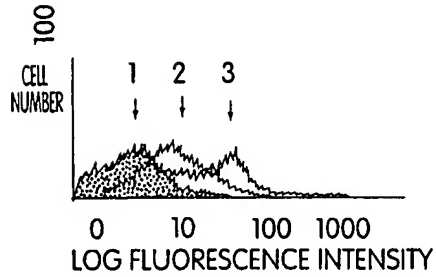


Fig. 2D

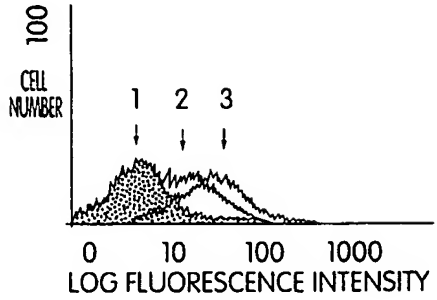


Fig. 2E

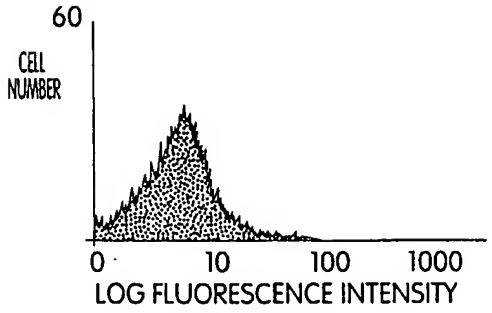


Fig. 3A

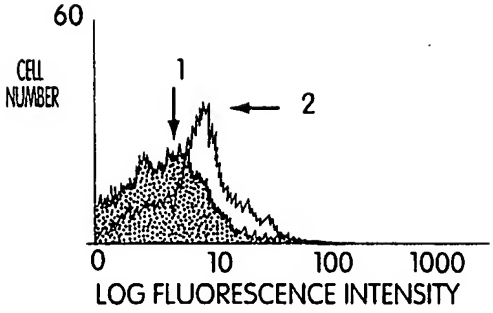


Fig. 3B

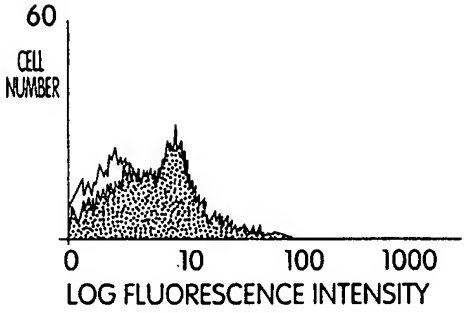


Fig. 3C

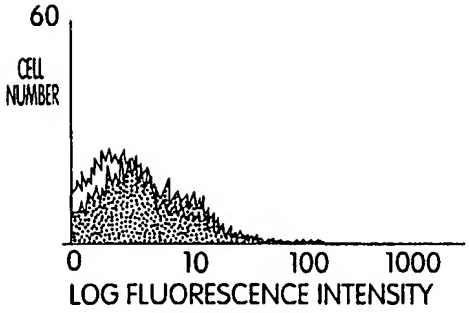


Fig. 3D

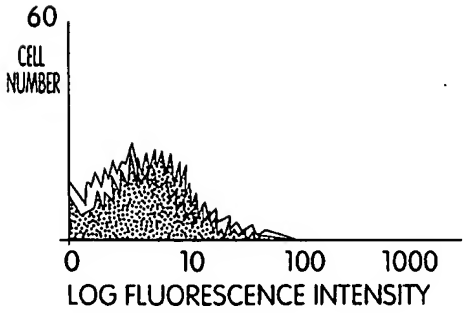


Fig. 3E

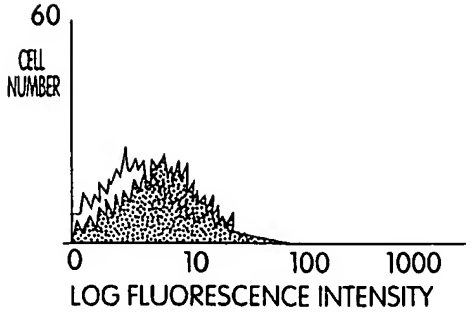
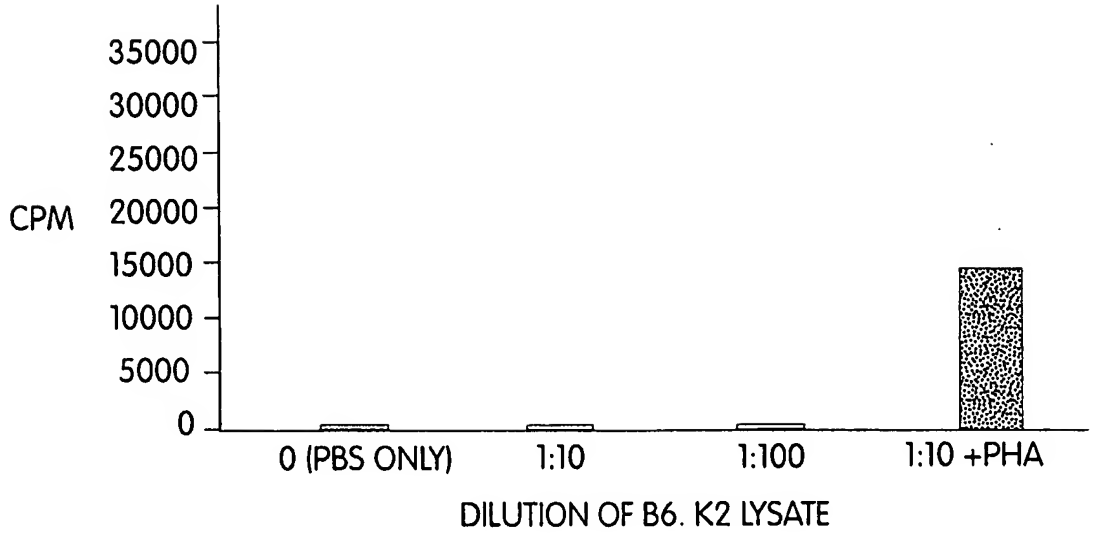


Fig. 3F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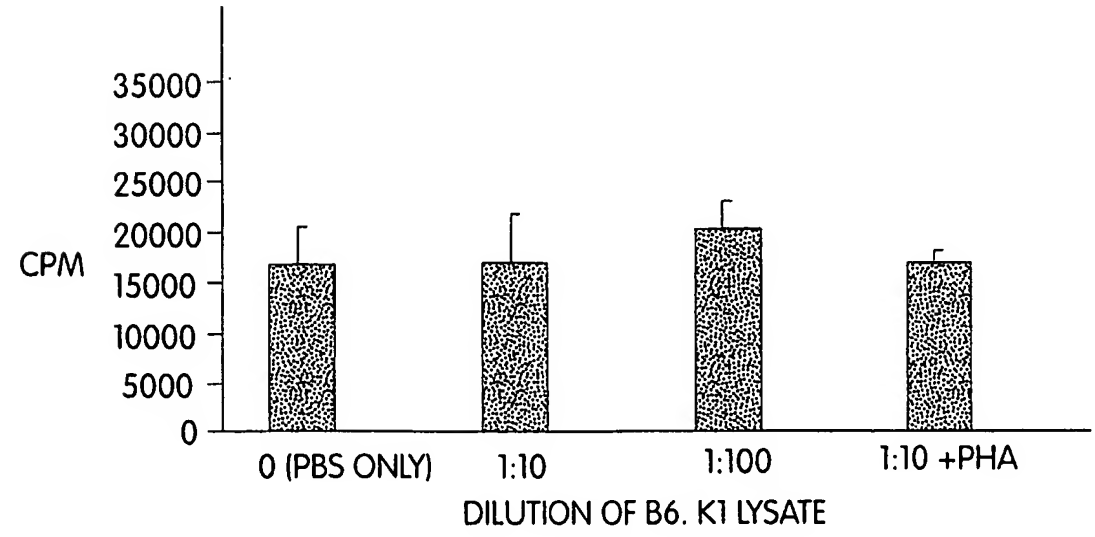
25000
20000
15000
10000
5000
0
CPM
0 (PBS ONLY)
1:10
1:100
1:10 +PHA
DILUTION OF B6. K2 LYSATE

Fig. 4A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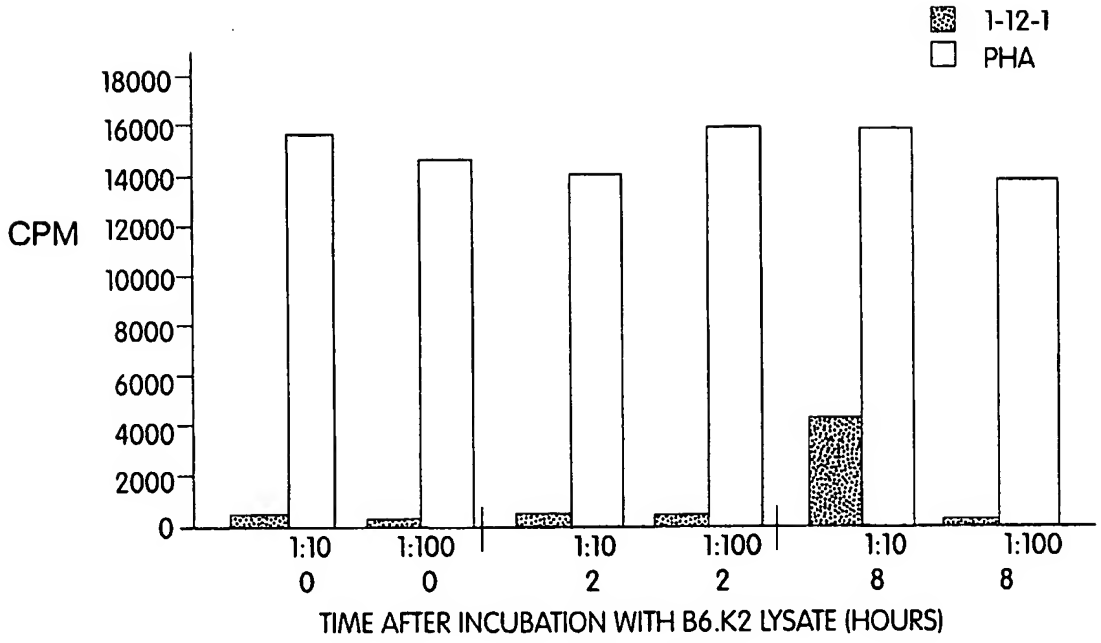
30000
25000
20000
15000
10000
5000
0
CPM
0 (PBS ONLY)
1:10
1:100
1:10 +PHA
DILUTION OF B6. K1 LYSATE

Fig. 4B


1-12-1
PHA
CPM
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16000
14000
12000
10000
8000
6000
4000
2000
0
1:10 0
1:100 0
1:10 2
1:100 2
1:10 8
1:100 8
TIME AFTER INCUBATION WITH B6.K2 LYSATE (HOURS)

Fig. 5

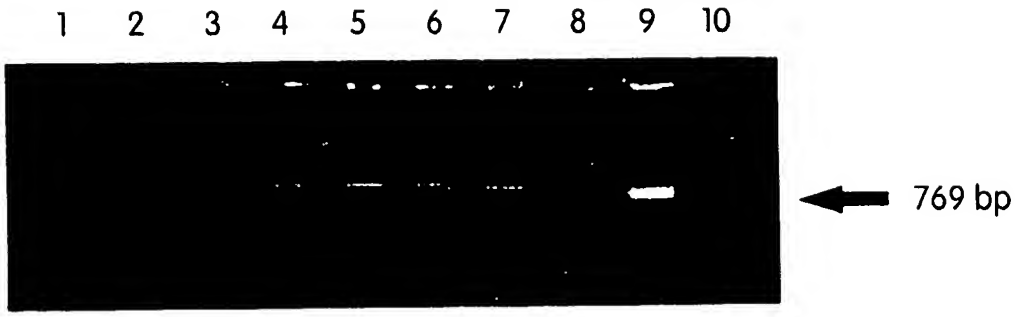

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15124

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12M 1/00; C12P 33/00; C12Q 1/68; G01N 33/53
US CL :435/287.1, 52, 6, 7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/287.1, 52, 6, 7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, STN, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAYLOR et al. Complement-binding Proteins are Strongly Expressed by Human Preimplantation Blastocysts and Cumulus Cells as Well as Gametes. 1996, Vol. 2, Number 1, pages 52-59, see entire document.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
05 NOVEMBER 1998	17 DEC 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	LIN SUN-HOFFMAN
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Form PCT/ISA/210 (second sheet)(July 1992)*